Journal of Forensic and Legal Medicine 22 (2014) 7–9

Contents lists available at ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/locate/jflm

Original communication

# The clavicle bone as an alternative matrix in forensic toxicological analysis

Ioanna Vardakou, PhD Scientific Associate, Sotiris Athanaselis, PhD Associate Professor, Constantinos Pistos, PhD Assistant Professor, Stauroula Papadodima, MD, PhD Assistant Professor, Chara Spiliopoulou, MD, PhD Professor, Konstantinos Moraitis, PhD Assistant Professor *

*Department of Forensic Medicine and Toxicology, Faculty of Medicine, University of Athens, 75 M. Asias Street, Athens 11527, Greece*

ARTICLE INFO

*Article history:*
Received 20 November 2012
Received in revised form
24 October 2013
Accepted 25 November 2013
Available online 3 December 2013

*Keywords:*
Forensic toxicology
Opiates
Bone
Clavicle

ABSTRACT

Although human blood is the reference medium in the field of forensic toxicology, alternative matrices may be required when traditional specimens are not available, especially in the investigation of cases involving decomposing remains. Clavicle bone may provide an appropriate sample of choice since it can easily be obtained at autopsy after the removal of the breastplate for the inspection of the thoracic viscera. To the author's knowledge, this is the first time that clavicle bone is used as an alternative matrix for the detection of drugs. The present study aimed to investigate the suitability of clavicle bone as an alternative matrix for the detection of opiates. Opiates were assayed using a gas chromatography-mass spectrometry in the selected ion monitoring mode. Morphine-d6, codeine-d6 and 6-MAM-d3 were used as internal standards for the determination of morphine, codeine and 6-MAM, respectively. A GC/MS method was developed and validated for the determination of opiates in clavicle samples. Morphine, codeine and 6-MAM were successfully separated in spiked samples allowing for their detection at low levels without interferences from the matrix. Chromatographic run time was 11 min and the tested linearity ranged from 5 to 500 ng/g ($r2 > 0.99$) for all analytes. The method was further applied in clavicle samples of drug-related cases. Its validation parameters and the application of the developed method in clavicle samples from drug addicts, prove its suitability for the detection of opiates and potentially other drugs.



## 1. Introduction

In medicolegal death investigations, routine specimens collected at autopsy for toxicological testing include blood (typically from central or peripheral vessels), urine, bile, vitreous humor, stomach or gastric contents, and visceral organs such as liver, brain, spleen, and lung.[1] In cases where the body has undergone significant decomposition, skeletonization or fragmentation, the analysis of bone samples may provide the only source of toxicological information.[2]

In the last decade, there have been a number of reports describing analysis of drugs in bone.[3–9] A common occurrence within these reports is the use of only a small number of different bone samples. Among those examined have been femoral mid-diaphysis,[3] as well as vertebral and pelvic bone samples.[1,4,7] To the author's knowledge, this is the first time that clavicle bone is used as an alternative matrix for the detection of drugs.

The present study aimed to investigate the suitability of clavicle bone as an alternative matrix for the detection of opiates. Our future prospects are to further investigate the suitability of the clavicle bone for the determination of other drug groups.

## 2. Material and methods

### 2.1. Material

Reference standards of morphine, codeine, and 6-MAM, morphine-$d_6$, codeine-$d_6$ and 6-MAM-$d_3$ were purchased from LGC-Promochem (Molsheim, France). Analytical- or HPLC-grade solvents (methanol, ethyl acetate, dichloromethane, isopropanol, ammonium hydroxide) were purchased from Merck (Darmstadt, Germany). Pentafluoropropionic anhydride (PFPA) 99% was purchased from Fluka (Steinheim, Germany). Bond-Elut Certify columns, containing a mixed-mode bonded silica SPE extraction

* Corresponding author. Tel.: +30 210 7462426; fax: +30 210 7706868.
*E-mail address:* kmoraitis@med.uoa.gr (K. Moraitis).

1752-928X/$ – see front matter © 2013 Elsevier Ltd and Faculty of Forensic and Legal Medicine. All rights reserved.
http://dx.doi.org/10.1016/j.jflm.2013.11.012

support with hydrophobic chains (octylsilane, n-C8) and strong cation-exchange moieties (benzenesulphonylpropylsilane), were provided by Varian (Houten, Netherlands). Midshaft clavicle samples were collected from autopsied cases referred to us from the Public Prosecutor's Office of District Court. The samples were extracted and analyzed according to the developed methodology.

### 2.2. Equipment

The analysis of the clavicle extracts was performed on a Shimadzu model GC-2010 gas chromatographer interfaced with a Shimadzu QP 2010S MS detector and equipped with a Shimadzu AOC-20i autosampler system. The separation of analytes was carried out using a cross-linked DB-1MS capillary column (12 m × 0.20 mm i.d., 0.33 μm film thickness). Helium was used as carrier gas at 1 mL/min flow rate. The MS was operated in electron impact (EI) ionization and selective ion monitoring (SIM) mode for the quantitation of all analytes.

A vortex (Chiltern, Model MT 19) set at speed 4, was used for the mixing of samples and standards. The pH-meter used was a 691 digital model pH-meter (Metrohm, Switzerland) with a glass combination electrode. Evaporation of the samples was performed under nitrogen using an evaporating device (Reacti-Vap Pierce, Model 18780, Rochford, IL). Centrifugation was performed using a centrifuge (Sigma 4K10, Germany). Water was deionized and further purified by means of a Milli-Q Plus water purification system, Millipore SA (Molsheim, France).

### 2.3. Chromatographic conditions

For the chromatographic separation of the analytes, an internal method that was validated in our laboratory was used for the determination of morphine, codeine, 6-MAM. The developed GC method was optimized for column temperature program, flow rate of carrier gas and temperatures of injector, ion source, and interface. The optimized GC conditions were as follows: initial temperature of 100 °C for 1 min, increased to 300 °C at 25 °C/min and held for 2 min giving a total run time of 11 min. Injector port and detector temperature were set at 260 °C and 280 °C, respectively. Analytes were identified using the ions of *m/z* **414**, 577 and 430 for morphine, *m/z* **282**, 445, 577 for codeine and *m/z* **414**, 473 and 361 for 6-MAM.

### 2.4. Calibrators and quality control samples

Three separate stock solutions of morphine, codeine and 6-MAM in methanol containing 1.0 mg/mL were prepared and stored at −20 °C. Appropriate dilutions with methanol yielded working standard solutions containing all three analytes. Combined working solutions containing morphine, codeine and 6-MAM were prepared with appropriate dilutions from stock standard solutions in absolute methanol. The concentrations of these solutions ranged between 100 and 10.000 ng/mL for each compound. Drug-free clavicle bone was soaked overnight in methanol, and subsequently methanol was screened to ensure that it was free of endogenous interference at the retention times of the analytes.

Fifty μL aliquot of the appropriate mixed working standard solution was spiked on 1 g of drug-free clavicle and then soaked in 1000 μL of methanol. Calibration samples were prepared freshly every day over a final concentration range of 5, 10, 75, 150, 500 ng/g for all three analytes. Quality control samples were prepared at three concentration levels (15, 200, and 400 ng/g). Calibration standard solutions and quality control samples were prepared from separate stock solutions prepared with separate weighing of the analyte.

### 2.5. Sample preparation

For the isolation of the analytes from clavicle samples an internal method for the determination of opiates in human urine was applied. This method was optimized and validated for the determination of morphine, codeine and 6-MAM in clavicle bone. Midshaft clavicle samples were harvested from autopsied cases referred to us from the Public Prosecutor's Office of District Court. Clavicle samples were mechanically cleaned of any overlying soft tissues using a scalpel or tissue scissors. The samples were subsequently rinsed with deionized water until the wash was clear and free of debris and air-dried at room temperature. The samples were then sectioned into 2 mm pieces by means of a band saw. The clavicle bone was weighted (1 g) and spiked with 50 μL of appropriate mixture of all analytes and 50 μL of the mixture of internal standards, and soaked in 1000 μL methanol. In each calibration and quality control sample, 50 μL of appropriate mixture of all analytes and 50 μL of the mixture of internal standards, were added. The clavicle bone remained in methanol overnight. The solvent was evaporated to dryness under a gentle stream of $N_2$ and reconstituted with 1 mL of ethyl acetate. All analytes and internal standards were extracted into 3 mL of phosphate buffer (pH 6.0, 1 M) while the sample was vortex mixing. Samples were stirred for 10 min and centrifuged at 4000 rpm for 10 min.

The supernatant organic layer was removed and the aqueous layer applied to the extraction columns, preconditioned with methanol and deionized water, 3 mL of each, and 1 mL of phosphate buffer (pH 6.0, 113 mM). The samples were passed through the cartridges at a flow rate of approximately 1.0 mL/min. The SPE columns were then washed subsequently with 3 mL of deionized water, 1 mL acetate buffer (pH 4.0, 100 mM), and 3 mL methanol, and drained under maximum vacuum for 5 min. The analytes were eluted twice with 1.5 mL of freshly prepared mixture of dichloromethane:isopropanol:ammonium hydroxide (85:15:2, v/v/v). The elution solvent was evaporated to dryness under a gentle stream of $N_2$. Dry extracts were derivatized with 50 μL PFPA under 70 °C for 30 min. The derivatized extract was finally evaporated to dryness and reconstituted with 50 μL of ethyl acetate. A 1 μL aliquot was injected into the chromatographic system.

### 2.6. Method validation

The following criteria were used to evaluate the GC/MS method according to FDA[10] and ICH[11] guidelines: selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, absolute recovery, and robustness. Selectivity, linearity, precision and accuracy of the method were validated through three analytical runs at three different days. The main validation parameters are presented in Table 1 while the compounds tested for the specificity of the method are presented in Table 2.

### 2.7. Application in real cases

The method was further applied in three clavicle bone samples from drug addicts and proved to be suitable for a quantitation of all

**Table 1**
Validation parameters of the analytical method ($n = 3$).

| Opiate | Equation of calibration curves | $R^2$ | LOD (ng/g) | LOQ (ng/g) | Rt (min) | %REC |
|---|---|---|---|---|---|---|
| Morphine | $s = 3.2 \times 10^{-3}\,C + 0.27 \times 10^{-3}$ | 0.991 | 0.61 | 1.83 | 9.95 | 96.90 |
| Codeine | $s = 9.2 \times 10^{-3}\,C + 2.6 \times 10^{-3}$ | 0.996 | 1.20 | 3.63 | 10.32 | 92.20 |
| 6-MAM | $s = 2.4 \times 10^{-3}\,C - 0.18 \times 10^{-3}$ | 0.998 | 0.47 | 1.40 | 10.66 | 92.68 |

**Table 2**
Compounds studied for interferences ($C = 250$ ng/g).

| | |
|---|---|
| Δ9-THC | Olanzapine |
| 11-carboxy-Δ9-THC | Thioridazine |
| Cocaine | Haloperidol |
| Ecgonine methylester | Pipamperone |
| Diltiazepam | Trifluoperazine |
| Nordiazepam | Perphenazine |
| Temazepam | Sulpiride |
| Alprazolam | Chlorpromazine |
| Nitrazepam | Carbamazepine |
| Midazolam | Venlafaxine |
| Flurazepam | Mirtazapine |
| Oxazepam | Penfluridole |
| 7-Aminoflunitrazepam | Paracetamol |
| Clobazam | Phenobarbital |
| Chlordiazepoxide | |

**Table 3**
Quantitation of opiates in biological samples.

| Case | Sex | Age | Cause of death | Biological sample | $C_{morphine}$ | $C_{codeine}$ | $C_{6MAM}$ |
|---|---|---|---|---|---|---|---|
| 1 | Male | 25 | Acute myocarditis | Clavicle bone[a] | 110.42 | 17.06 | ND |
| | | | | Blood[b] | 77.81 | 14.09 | ND |
| | | | | Urine | + | + | ND |
| 2 | Male | 25 | Opiates poisoning | Clavicle bone[a] | 186.10 | 17.81 | ND |
| | | | | Blood[b] | 187.50 | 20.83 | ND |
| | | | | Urine | + | + | + |
| 3 | Male | 53 | Acute myocarditis | Clavicle bone[a] | ND | 5.48 | ND |
| | | | | Blood[b] | 21.88 | ND | ND |
| | | | | Urine | N/A | N/A | N/A |

Urine (+): Positive by GC/MS.
ND: not detected.
N/A: not applicable.
[a] Concentration in clavicle bone expressed in ng/g.
[b] Concentration in whole blood expressed in ng/mL.

analytes during the investigation of forensic cases, offering high selectivity and sensitivity. The results of all three cases obtained from clavicle bone and other biological samples (blood, urine) are presented in Table 3.

## 3. Discussion

In forensic toxicology, although blood is the reference sample for qualitative and quantitative interpretation various tissues may be available for analysis according to the circumstances of death. In medicolegal cases involving skeletonized remains bone may be the only alternative tissue for toxicological investigation. Several factors influence the deposition of drugs in this matrix including acute versus chronic exposure, distribution at the time of death, site of bone collection and bone type, and physicochemical characteristics of the drug. Drugs with short half lives or those inherently unstable, such as 6-acetylmorphine, may not be stored in bone. In addition, polar metabolites and highly protein-bound compounds may be transferred from blood to bone to a lesser extent.[1]

This survey focuses on the use of clavicle bone as an alternative matrix in forensic toxicological analysis. Bones in general present several advantages, such as protection against contamination and decomposition since they are very resistant to postmortem disintegration and fragmentation in comparison to other tissues of the human body. Moreover bone is a highly vascular tissue and contains a large amount of lipids within bone marrow. Especially for the clavicle bone, this part of the human skeleton is easy to detach during autopsy after the removal of the breastplate for the inspection of the thoracic viscera, without compromising the integrity of the body.

The use of clavicle bone as an alternative matrix for opiates detection was attempted. For this purpose a GC/MS method for the determination of three opiates in clavicle was developed and validated. The validation parameters as well as its application to clavicle samples from drug addicts prove its suitability for potential use during the forensic investigation.

It can be concluded that the use of clavicle bone is of great potential interest in forensic casework where drug involvement may be an issue and where routine toxicological specimens are not available. Similar methods could be also developed for the detection of other substances in clavicle bone.

## Ethical approval

None.

## Funding

None.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

## Acknowledgments

The authors would like to express their gratitude to mortuary technician Mr Dimitris Mourlas for his help in obtaining the bone samples. We also wish to thank Ms Christina Paraskeuvopoulou for her technical support in the preparation of the specimens.

## References

1. McGrath KK, Jenkins AJ. Detection of drugs of forensic importance in postmortem bone. *Am J Forensic Med Pathol* 2009;**30**:40–4.
2. Watterson J. Challenges in forensic toxicology of skeletonized human remains. *Analyst* 2006;**131**:961–5.
3. McIntyre IM, King CV, Boratto M, Drummer OH. Postmortem drug analyses in bone and bone marrow. *Ther Drug Monit* 2000;**22**:79–83.
4. Horak EL, Jenkins AJ. Postmortem tissue distribution of olanzapine and citalopram in a drug intoxication. *J Forensic Sci* 2005;**50**:679–81.
5. Kudo K, Sugie H, Syoui N, Kurihara K, Jitsufuchi N, Imamura T, et al. Detection of triazolam in skeletal remains buried for 4 years. *Int J Legal Med* 1997;**110**:281–3.
6. VandenBoer TC, Grummett SA, Watterson JH. Utility of immunoassay in drug screening in skeletal tissues: sampling considerations in detection of ketamine exposure in femoral bone and bone marrow following acute administration using ELISA. *J Forensic Sci* 2008;**53**:1474–82.
7. Watterson JH, Botman JE. Effects of tissue type and the dose-death interval on the detection of acute diazepam exposure in bone and marrow with solid-phase extraction, ELISA and liquid chromatography tandem mass spectrometry. *J Forensic Sci* 2009;**54**:708–14.
8. Desrosiers NA, Betit CC, Watterson JH. Microwave assisted extraction in toxicological screening of skeletal tissues. *Forensic Sci Int* 2009;**188**:23–30.
9. Lafreniere NM, Watterson JH. Detection of acute fentanyl exposure in fresh and decomposed skeletal tissues. *Forensic Sci Int* 2009;**185**:100–9.
10. CDER. *Guidance for industry. Bioanalytical method validation*. U.S. Department of Health and Human Services, Food and Drug Administration; 2001.
11. ICH. *Harmonized tripartite guideline*In *Validation of analytical procedures text and methodology*; 2005. Q2 (R1).